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Permeable trypanosome cells as a model system for transcription and trans-splicing

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ABSTRACT

We have established conditions for *Trypanosoma brucei* permeable cells to study transcription and trans-splicing. We found that the concentration of monovalent and, to a lesser extent, divalent ions plays a critical role for the expression of a number of different genes. Most remarkably, the synthesis of the spliced leader (SL) RNA was optimal at 20 mM KCl, whereas higher potassium concentrations were inhibitory. In addition, $MgCl_2$ concentrations above 3 mM led to the accumulation of a 3' end shortened SL RNA species, which has been previously reported not to participate in trans-splicing (20). Using conditions optimal for the synthesis of the SL RNA, we observed accurate trans-splicing of newly-synthesized α-tubulin RNA. Moreover, we detected the SL intron both joined to high molecular weight RNAs in the form of branched Y-structures and as a free linear molecule, which rapidly turned over. Furthermore, ionic concentrations that inhibit the synthesis of the SL RNA produced exclusively unspliced α-tubulin RNA, thus demonstrating that transcription and trans-splicing can be uncoupled.

INTRODUCTION

In *Trypanosoma brucei*, as well as in several other protozoa of the order *Kinetoplastida*, the biogenesis of mRNA is achieved through unusual mechanisms. Protein coding genes appear to be transcribed as part of complex polycistronic (1,2) and polygenic (3) transcription units to generate RNA primary transcripts that contain more than one protein coding region. The formation of the 5' end of mature mRNA molecules is achieved *via* trans-splicing, an RNA processing reaction that entails the joining of a 39 nt leader sequence (spliced leader (SL) sequence) to the body of the mRNA which codes for different proteins (4–6). In *T. brucei* the substrates for trans-splicing are the 140 nt long spliced leader RNA (SL RNA), which provides the SL sequence and the 5' splice site (7–9), and pre-mRNAs, which contain one or several 3' splice sites (10–18). Several observations suggest that the mechanisms of cis-splicing of intervening sequences and trans-splicing are closely related. Most compelling has been the identification in steady-state RNA of linear branched molecules consisting of the SL intron (the 100 nt downstream from the SL sequence in the SL RNA) joined *via* a 2'-5' linkage to high molecular weight RNAs (4–6); these molecules would be analogous to the intron-exon lariat intermediates that form during the first step of the cis-splicing reaction (19). Since these findings were published three years ago, not much progress has been made towards understanding the mechanism of trans-splicing. This is because no trypanosome system competent for assaying transcription and trans-splicing *in vivo* or *in vitro* has been established.

To overcome these limitations we decided to explore permeable trypanosome cells as a model system to study transcription and trans-splicing. In a previous report we have shown that, after brief exposure to the detergent lysolecithin, cultured procyclic trypanosomes become permeable to nucleotide triphosphates and synthesize RNA (3). However, at that time we were unable to obtain a linear rate of RNA synthesis for longer than 5 minutes or to detect trans-splicing activity. By systematically varying several parameters of the permeabilization protocol and incubation conditions we have now succeeded in obtaining a cell preparation that accurately transcribes trypanosome genes and maintains the ability to trans-splice newly synthesized SL RNA and pre-mRNAs. Thus, permeable cells constitute a suitable system to investigate the physiology and regulation of mRNA biogenesis in trypanosomes.

MATERIALS AND METHODS

Preparation of permeable cells and conditions for RNA synthesis

To obtain high levels of trans-splicing activity it was essential that the cell culture density was well below saturation and that the cells were frequently diluted to keep the number of dead cells to a minimum. In addition, it was important to carry out the permeabilization procedure as fast as possible. Procyclic trypanosome forms of the YTaT strain were grown in culture as described previously (3) but using 10% fetal calf serum. To prepare permeable cells, mid-logarithmic phase trypanosomes ($5-7\times10^6$ cells/ml) were harvested by centrifugation at room temperature in a table top centrifuge (IEC, model CL centrifuge) at top speed for 5 minutes. Cell pellets were washed twice with

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1 ml of culture medium without serum and collected by centrifugation for 30 seconds in a variable speed Hill minicentrifuge at 4500 rpm at room temperature. This was followed by two additional washes using buffer A (150 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, 20 mM HEPES-KOH [pH 7.9], 1 mM DTT and 10 μg/ml leupeptin). Finally, cells were resuspended in buffer A at a density of 6×10^8 cells/ml, divided into 400 μl aliquots, chilled on ice for 5 minutes and then L-α-lysophosphatidylcholine, palmitoyl (stock solution in water; Sigma) was added to a final concentration of 500 μg/ml. After a 1 minute incubation on ice, two volumes of buffer A (kept at room temperature) were added and the cells were recovered by centrifugation as above and washed once more in the same buffer. Each cell pellet derived from 2.4×10^8 cells was resuspended in a total volume of 100 μl of buffer A. At this point the cells can be kept on ice for up to 30 minutes without appreciable loss of activity. To initiate transcription an equal volume of transcription cocktail was added to give the following final concentrations: 2 mM ATP, 1 mM GTP, 1 mM CTP, 75 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 10 μg/ml leupeptin, 25 mM creatine phosphate, 0.6 mg/ml creatine kinase and 500 μCi/ml [α-^{32}P]UTP (3000 Ci/mmol, Amersham International). Cells were incubated at 28°C for the required period of time, and then DNAse I was added to 200 μg/ml and incubation was continued for 30 seconds at room temperature. Cells were lysed by adjusting the reaction mixture to 1% SDS, 1 mg/ml proteinase K and 20 mM EDTA (pH 8.0). After incubation at 55°C for 15–30 minutes, nucleic acids were precipitated with 1 volume of 4 M ammonium acetate (pH 6.0) plus three volumes of ethanol, recovered by centrifugation at room temperature for 15 minutes in a microcentrifuge and rinsed with 70% ethanol. This treatment resulted in the removal of more than 95% of unincorporated nucleotide triphosphates. Routinely, $1-2\times10^6$ cpm were incorporated into RNA in 10–15 minutes per 10^7 cells.

RNA analysis

Fractionation of radiolabelled RNA by electrophoresis was carried out through 6% polyacrylamide-7 M urea gels. After electrophoresis the gels were dried and exposed to X-ray film. RNAse mapping with biotinylated antisense α-tubulin RNA probes was carried out essentially as described (3), except that only one cycle of hybrid enrichment by streptavidin-agarose was performed. For DNA dot blot analysis the following trypanosome plasmid clones were used: pSPSL2/65, contains a cDNA copy of the SL RNA from nucleotide 7 to nucleotide 128, cloned into the vector pSP65 (C.T., unpublished data); pTB7SL contains a 600 nt genomic fragment coding for *T. brucei gambiense* 7SL RNA, cloned into pT3T7 (E.U., unpublished data); pTUBS is a genomic clone in pT3T7 containing the 1.1 kb *EcoRI-SalI* fragment of the α- and β-tubulin repeat (14, and our unpublished data); pr2 and pr7 are two genomic *EcoRI* fragments spanning sequences coding for the two large ribosomal RNAs (V.Asher and E.U., unpublished data); p84A3 is a genomic clone of 500 bp which contains part of the U4 snRNA coding region (C.T., unpublished data). Plasmid DNAs (5 μg/dot) were incubated at 55–60°C in 0.3 N NaOH for 15 minutes, diluted with one volume of 2 M ammonium acetate and bound onto nitrocellulose filters using a S&S manyfold filtration apparatus. Hybridizations were carried out at 68°C for 16 hrs in 5× SET (1×SET= 150 mM NaCl, 30 mM Tris-HCl [pH 7.8], 1 mM EDTA), 10× Denhardt's, 1% SDS, 100 μg/ml carrier tRNA and $10-20\times10^6$ cpm of radiolabelled RNA in a total volume of 2 milliliters. After hybridization the filters were extensively washed in 2× SET at 65°C, incubated with 20 μg/ml RNAse A in 2× SET for 30 minutes at 37°C, rinsed in 2× SET at 37°C and exposed to Kodak X-ray film.

RESULTS

Parameters affecting RNA synthesis in permeable trypanosome cells

Cultured procyclic cells of *Trypanosoma brucei rhodesiense* were permeabilized by exposure to the detergent lysolecithin following the general outline of the procedure reported previously (3), but with several modifications as described in Materials and Methods. To establish which parameters were critical for accurate and efficient RNA synthesis in permeable trypanosomes, we initially measured the extent and kinetic of incorporation of radiolabelled UTP into TCA-insoluble material and analyzed by gel electrophoresis the quality of the RNA synthesized (i.e. discrete RNA bands and size) under a variety of conditions. In the course of these experiments we made the following observations (data not shown). First, in order to maintain a linear rate of RNA synthesis for at least 15–20 minutes, glycerol must be excluded from the incubation cocktail and creatine phosphokinase plus creatine phosphate must be present. If glycerol was included, as in our original protocol (3), the ATP pool was rapidly depleted even in the presence of an energy regenerating system leading to a complete halt of RNA synthesis. We tested a number of compounds to replace glycerol in the incubation cocktail including polyethylene glycol (PEG), sucrose and polyvinyl alcohol (PVA). At the concentrations tested all compounds supported linear incorporation of UTP but the relative efficiency of RNA synthesis decreased in the following order: 75 mM sucrose or 3% PVA > 1 M sucrose >> 10% PEG. However, the RNA synthesized in 75 mM sucrose was qualitatively much better than the one obtained in the presence of PVA both in terms of overall size and presence of discrete RNA species. Second, the incorporation of radiolabelled UTP was proportional to the cell number, if we used between 1.2×10^8 and 1.2×10^9 cells/ml in the transcription reaction. Increasing the cell number over 1.2×10^9/ml resulted in a reduction on a per cell basis of the ability to incorporate the label and in an overall shortening of the RNA chains. Third, acetate and chloride salts of potassium and magnesium were equally effective at supporting RNA synthesis.

Potassium and magnesium requirements for SL RNA synthesis

Because we wished to establish optimal conditions for both transcription and for trans-splicing, we first concentrated on identifying optimal ionic conditions for SL RNA synthesis. In initial experiments we made the fortuitous observation that the accumulation of SL RNA was dramatically reduced at KCl concentrations above 20 mM. We therefore determined the optimum $MgCl_2$ concentration for SL RNA synthesis in the presence of 20 mM KCl. Figure 1A shows the patterns of low molecular weight RNAs synthesized at five different $MgCl_2$ concentrations ranging from 1.5 mM to 12 mM. The maximal accumulation of intact SL RNA was observed at 3 mM $MgCl_2$ (the identity of the SL RNA was established by hybrid selection with cloned SL DNA, see below). At higher magnesium concentrations we observed an increase of a 130 nucleotide RNA species (indicated by an arrowhead). By site-directed cleavage

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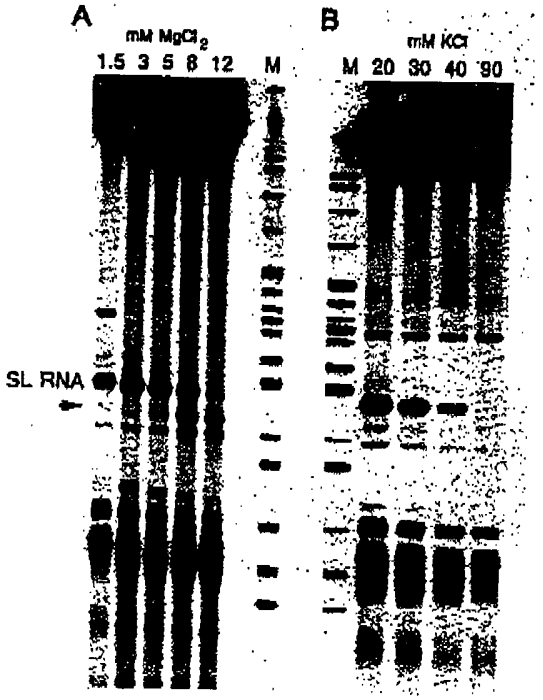

Figure 1. A: RNA synthesis: titration of magnesium ions. Radiolabelled RNA was synthesized for 8 min. in permeable cells using variable amounts of $MgCl_2$, as indicated above each lane (mM), and a fixed concentration of KCl (20 mM). Aliquots from each reaction were fractionated by electrophoresis on a 6% polyacrylamide-7 M urea gel and ^{32}P-RNA was detected by autoradiography. B: RNA synthesis: titration of potassium ions. RNA was synthesized for 15 min. using variable amounts of KCl as indicated and a constant $MgCl_2$ concentration of 3 mM. The position of the SL RNA is indicated and a solid arrowhead indicates the position of the SL RNA 130 (see text for details). M, end labelled *Msp*I fragments of pBR322 as molecular weight markers. A prominent RNA migrating above the SL RNA can be seen in (B), whereas this RNA is absent under identical conditions in (A). This occurred in a few experiments and at present we do not have an explanation for this phenomena, nor do we know the identity of this RNA.

with ribonuclease H and complementary oligonucleotides, we have determined that the predominant component of the 130 nt band is SL RNA shortened at the 3′ end (data not shown). Most likely this RNA corresponds to an SL RNA species of similar size and structure that was previously identified in steady-state RNA and does not seem to be a substrate for trans-splicing (20).

Having established that the addition of 3 mM $MgCl_2$ to the transcription mixture was optimal for the accumulation of intact SL RNA, we re-investigated the effect of varying the KCl concentration (Figure 1B). Increasing the potassium concentration above 20 mM resulted in a progressive decline in the amount of SL RNA accumulation. At 90 mM KCl we detected very little SL RNA. In addition, no substantial amount of the 3′ end shortened SL RNA of 130 nt accumulated.

Potassium and magnesium ions modulate the synthesis of a variety of cellular RNAs

Next we quantitated the accumulation of a number of different transcripts over the range of ionic conditions shown in Figures 1A and 1B. In this experiment, cloned trypanosome DNAs coding for large ribosomal RNAs, α- and β-tubulin mRNAs, SL RNA, U4 snRNA and 7SL RNA were immobilized onto nitrocellulose filters and hybridized to total radiolabelled RNA synthesized under various ionic conditions. After hybridization and washing of the filters, the radioactivity associated with each dot was determined by liquid scintillation counting (Figure 2A and 2B). The synthesis of all transcripts tested, with the exception of the large ribosomal RNAs, was inhibited at KCl concentrations above 20 mM (Figure 2A). The extent of inhibition was variable for different gene products (Figure 2A). Most dramatic was the response of the SL RNA whose accumulation was reduced 80% by raising the potassium concentration from 20 to 40 mM. The U4 and 7SL RNAs behaved similarly to the SL RNA. Instead, the tubulin RNAs were the least sensitive to increments of potassium above 20 mM. Similar results were obtained for the calmodulin and ornithine decarboxylase mRNAs (data not shown). Finally, the accumulation of ribosomal RNAs was stimulated by increasing the potassium concentration up to 150 mM.

Variations in the magnesium concentration of the incubation cocktail affected the accumulation of different transcripts to various degrees (Figure 2B). The response curves of the tubulin, 7SL and U4 RNAs showed a rather broad optimum for magnesium around 4–8 mM. In contrast, the synthesis of the large ribosomal RNAs had a magnesium optimum which was above 8 mM. Finally, the total accumulation of the SL RNA sequences, that is the SL RNA plus the SL RNA 130 and perhaps other degradation products of the SL RNA, displayed a clear maximum at 5 mM magnesium. From these data we concluded that the ionic conditions that promoted the synthesis and accumulation of intact SL RNA were permissive for the accumulation of other gene products and in particular for mRNA transcripts, such as the α- and β-tubulin RNAs. At the optimal $MgCl_2$ concentration for the accumulation of intact SL RNA (3 mM), the tubulin RNAs were still quite efficiently synthesized: the accumulation was reduced by only 20% relative to the maximum. On the other hand, the synthesis of the large ribosomal RNAs followed a trend that was opposite to the one of all other RNAs tested, being maximal at high potassium and high magnesium concentrations.

Inhibition of transcription by α-amanitin

To confirm that the normal physiology of RNA synthesis was maintained in permeable trypanosome cells, we determined the effect of the elongation inhibitor α-amanitin on the transcription of various genes (Figure 3), using ionic conditions that are optimal for SL RNA synthesis and accumulation (3 mM $MgCl_2$ and 20 mM KCl). The transcription of the ribosomal genes was insensitive to high concentrations of the inhibitor (500 μg/ml), whereas the synthesis of tubulin RNAs was 90% inhibited at the lowest α-amanitin concentration tested (5 μg/ml). The synthesis of the SL RNA was 50% inhibited at 15 μg/ml α-amanitin. These results are in agreement with data reported earlier using run-on transcription assays in isolated trypanosome nuclei (21).

Evidence for trans-splicing

Detection of the SL exon and SL intron

To determine if trans-splicing was taking place in our permeable cell system, we first determined the structure of newly-synthesized SL RNA sequences by RNAse protection experiments. As probe we used an unlabelled antisense SL RNA which is complementary to nucleotides 7 to 128 of the SL RNA. A typical result of the RNAse mapping experiments is shown in Figure 4. We detected four fragments with approximate

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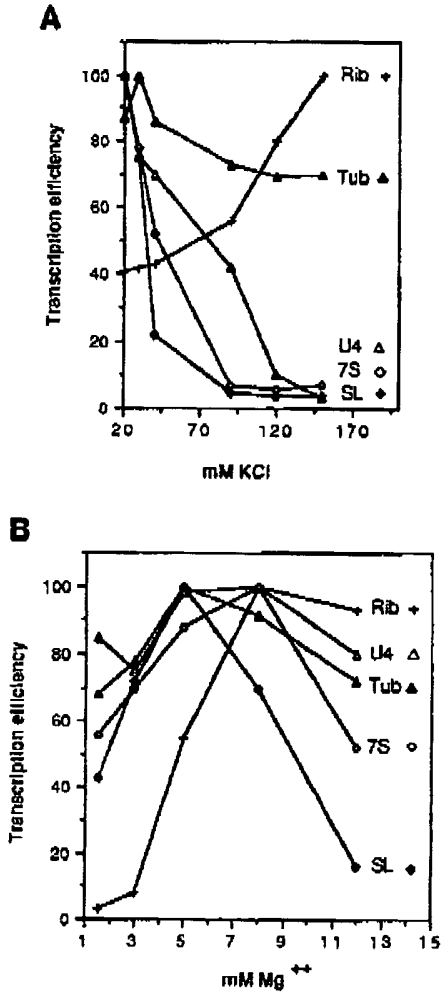

Figure 2. A: Quantitation of the synthesis of various cellular RNAs in the presence of variable amounts of KCl. ^{32}P-RNA from 1.2×10^8 cells was synthesized as described in Figure 1B and hybridized to dot blots of plasmid DNAs coding for the large ribosomal RNAs, SL RNA, α- and β-tubulin RNAs, 7SL RNA and U4 snRNA (see Materials and Methods for details). For each dot the amount of hybridized RNA was determined by liquid scintillation counting; for each gene the highest hybridization value was set at 100%, whereas the other values were expressed as percentages of that number. B: Quantitation of the synthesis of various cellular RNAs in the presence of variable amounts of $MgCl_2$. ^{32}P-RNA was synthesized as described in Figure 1A and quantitated as described in (A).

mobilities of 129, 123, 90, and 39 nt. The 123 nt RNA species had the correct size expected for protection of SL RNA which had not been cleaved at the 5′ splice site. The 129 nt RNA was longer than the fragment expected for full protection of the probe (123 nt) and may be derived from incomplete cleavage of intact SL RNA by ribonuclease A and T_1. This would be the case if the 129 nt SL RNA fragment has a fully methylated cap structure (in which the first four nucleotides adjacent to the cap are methylated; refs. 22–24), and therefore the ribonucleases would not be able to cleave the 5′ end sequence ($A_{me}A_{me}U_{me}C_{me}AA$) which is not represented in the probe. Indeed, a great proportion of the SL RNA made in permeable cells appeared to be capped as judged by 5′ end group analysis and by immunoprecipitation

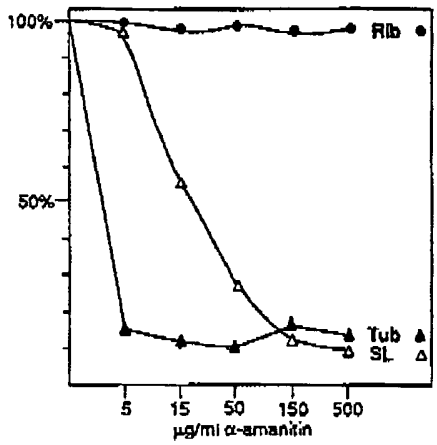

Figure 3. Inhibition of RNA synthesis by α-amanitin. Permeable trypanosomes were preincubated in buffer A for 5 minutes on ice in the presence of the indicated amounts of α-amanitin. Transcription was initiated by adding the remainder of the components and allowed to proceed for 15 minutes. The extent of RNA synthesis by the ribosomal, SL, and tubulin genes was determined by dot blot analysis as described in Figure 2A and is expressed as percentages of the value observed in the absence of the inhibitor.

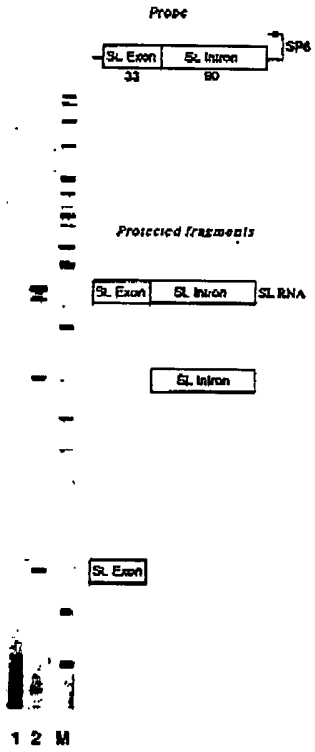

Figure 4. Fractionation of radiolabelled RNA fragments protected from RNAse digestion by the antisense SL RNA probe. Radiolabelled RNA from 6×10^6 cells was analyzed by RNAse mapping in the absence (lane 1) or presence (lane 2) of 200 ng of unlabelled SL antisense RNA essentially as described (3) except that 40% formamide was included in the hybridization solution and hybridization was carried out at 37°C. The products of digestion were analyzed by electrophoresis as described in Figure 1. M, molecular weight marker. The structures of the antisense SL RNA probe and of the expected products of RNAse digestion are shown on the right.

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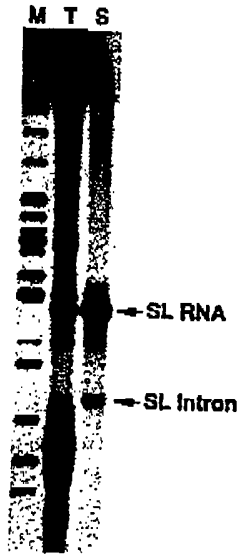

Figure 5. Electrophoretic fractionation of SL RNA sequences selected by hybridization to cloned SL DNA. Radiolabelled RNA from 5×10^7 cells was hybridized to plasmid DNA pSPSL2/65 (10 μg) immobilized onto a nitrocellulose filter as described in Materials and Methods. After hybridization and extensive washing, hybrids were eluted by boiling in 1 mM EDTA. Hybrid selected RNAs were fractionated as in Figure 1. T, total RNA; S, hybrid selected RNA; M, molecular weight marker.

with anti-7-methylguanosine cap antibodies (data not shown). Therefore, both the 123 and 129 SL RNA fragments represented SL RNA which had not been cleaved at the 5′ splice site. On the other hand, the 90 nt and 39 nt long SL RNA fragments had the sizes consistent with their identity as the SL intron and the SL exon, respectively. Also in this case the SL exon fragment (39 nt) was longer than predicted (33 nt) which was most likely due to incomplete cleavage by RNAse A and T_1 (see above).

A high proportion of the SL intron is present as a free linear molecule

To establish the origin of the SL intron fragments identified by RNAse mapping (see above) we first determined whether free SL intron accumulated in permeable trypanosome cells. This was accomplished by hybrid selection of newly-synthesized SL RNA sequences to cloned SL DNA immobilized onto a nitrocellulose filter, followed by electrophoretic fractionation of the hybridized material (Figure 5). Two distinct RNA species were selected: the most prominent one had the mobility of intact SL RNA (140 nt), whereas the other was approximately 100 nt long suggesting that this RNA was the free form of the SL intron. In addition, the SL DNA selected high molecular weight heterogeneous RNAs, which most likely represents mRNA molecules already joined to the SL sequence, and a cluster of narrowly spaced bands between 123 and 160 nt, whose identity is at present uncertain.

Proof for the identity of the free SL intron was obtained by direct sequence determination (Figure 6). For this experiment, we first compared the amount of putative free SL intron produced by incubation of permeable trypanosome cells with the amount of SL intron present prior to incubation. Total unlabelled RNA was isolated from equal numbers of cells and fractionated on a sequencing gel. RNA migrating at the position of the free SL intron (identified by running radiolabelled RNA in a parallel lane)

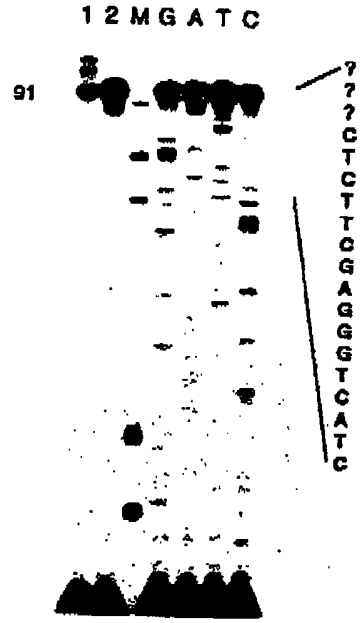

Figure 6. Sequence determination of the free SL intron. Total unlabelled RNA was isolated from equal numbers of permeable cells prior to the transcription assay (lane 1) and after the transcription assay (lane 2). RNA migrating at the position of the free SL intron was gel purified and subjected to primer extension analysis with 5′ end-labelled oligonucleotide SL-3′ which is complementary to nt 113–130 of the SL RNA. The extension products were fractionated as described in Figure 1. The size of the extension product is 91 nucleotides. Lanes G, A, T, and C, the free SL intron produced upon incubation of permeable cells was extended in the presence of appropriate dideoxynucleotide/deoxynucleotide triphosphate mixtures. The sequence generated is complementary to the SL RNA. M, end-labelled *MspI* fragments of pBR322.

was eluted and subjected to primer extension analysis using as a primer an oligonucleotide complementary to positions 113–130 of the SL RNA (SL-3′). Free SL intron should produce an extension product of 91 nucleotides. This experiment showed that the isolated RNAs generated extension products of identical mobilities with a size expected from the free SL intron (Figure 6, lanes 1 and 2). In addition, comparison of the amount of cDNAs produced by the two RNA samples (compare lanes 1 and 2) revealed that there was at least 10-times more free SL intron at the end of the incubation period (lane 2) as compared to the amount present at the beginning of the reaction (lane 1). This observation enabled us to sequence the free SL intron produced upon incubation of permeable cells using labelled oligonucleotide SL-3′ as a primer and dideoxynucleotide triphosphates. The nucleotide sequence of the extension product (lanes G, A, T, C) clearly demonstrated that this was indeed the free SL intron and that this RNA species terminated at the position of the splice junction.

The abundance of free SL intron was quite high considering the fact that it was easily detectable in total RNA (Figure 5, see also Figure 1A). This RNA species is most likely a by-product of trans-splicing and is derived from debranching forked SL intron pre-mRNA molecules (4–6,25). It is conceivable, therefore, that this molecule is destined to degradation. To follow the fate of free SL intron in permeable cells we did a continuous labelling experiment (Figure 7). Whereas the SL RNA continued to accumulate for 20 minutes, the amount of free SL intron peaked

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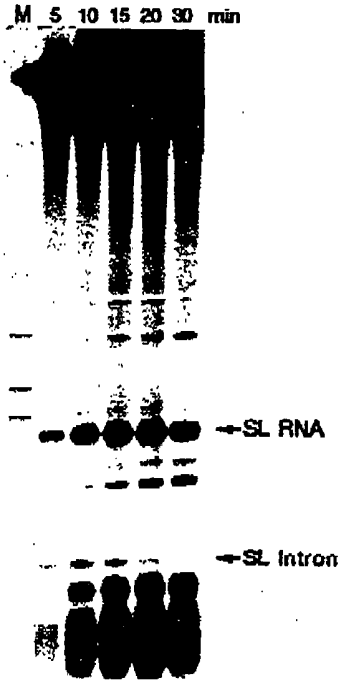

Figure 7. Time course of RNA accumulation. ^{32}P-RNA was synthesized for the indicated periods of time; RNA from 5×10^6 cells was fractionated on a 6% acrylamide-7M urea gel and detected by autoradiography. M, end-labelled *MspI* fragments of pBR322.

at 10 minutes and thereafter decreased and free SL intron was no longer detectable at 30 minutes. Therefore, under our experimental conditions the free SL intron turned over quite rapidly and this is in agreement with its identity as a terminal product of trans-splicing.

Identification of branched SL intron-high molecular weight RNA molecules

In steady-state RNA three different forms of the SL intron have been identified so far: free SL intron (5), branched SL intron-low molecular weight RNA molecules (26) and branched SL intron-high molecular weight RNA molecules (4–6). The latter of these molecules should be generated during the first step of trans-splicing, that is cleavage at the 5′ splice site of the SL RNA coupled to the joining of the 5′ end of the SL intron to a branch site sequence upstream from the pre-mRNA 3′ splice site. To further confirm that permeable trypanosomes were fully competent to perform all the predicted steps of trans-splicing, we analyzed high molecular weight RNAs for the presence of branched SL intron containing molecules. Radiolabelled RNA was synthesized, partially depleted of low molecular weight RNAs by precipitation with isopropanol and fractionated by sucrose density gradient centrifugation. Next, fractions enriched in high molecular weight RNAs were incubated in a HeLa cell S100 extract containing debranching activity (27) and the release of the SL intron was monitored by gel electrophoresis (Figure 8). Indeed, when this RNA, which *per se* did not contain detectable amounts of linear SL intron, was subjected to debranching by the human enzyme, RNA molecules with the electrophoretic mobility of linear SL intron were released. Under

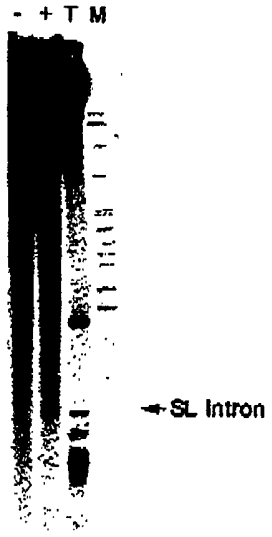

Figure 8. 'Debranching' of high molecular weight RNA. ^{32}P-RNA from 2.5×10^8 cells was partially depleted of low molecular weight RNAs by two rounds of precipitation with 0.5 volumes of isopropanol and sedimented through a 5%–20% sucrose density gradient as described (3). An aliquot of high molecular weight RNA was ethanol precipitated and incubated in the absence (lane labelled −) or in the presence (lane labelled +) of a HeLa cell S100 extract containing debranching activity (27). RNAs were deproteinized and fractionated by electrophoresis as described in Figure 1. T, input RNA; M, molecular weight marker.

our experimental conditions the amount of SL intron associated with branched molecules was much lower than the amount of free SL intron, since debranching of unfractionated RNA resulted in only a slight increase of the total amount of linear SL intron (data not shown). In conclusion, the identification in newly-synthesized RNA of the SL exon and of the free and branched forms of the SL intron implies that trans-splicing is actively taking place in permeable trypanosome cells.

Accurate trans-splicing of α-tubulin mRNA

We next investigated whether we could detect accurate 5′ end formation of RNAs by joining of the SL exon to mRNA sequences. As a model RNA we chose the α-tubulin mRNA because it is abundant and only one 3′ splice site is used for joining the SL sequence (14,28). As an assay we employed RNAse protection of newly transcribed RNAs using as probe an antisense RNA complementary to the mature 5′ end of the α-tubulin mRNA, which consists of the SL sequence joined to α-tubulin coding sequences (Figure 9). As diagrammed in the figure, if trans-splicing of α-tubulin mRNA is accurate, the probe should protect from RNAse digestion an RNA species of 164 nt. In contrast, unspliced α-tubulin sequences lacking the SL sequence should generate RNA molecules of 125 nt, that are 39 nt shorter than the trans-spliced product. For these experiments, RNA was synthesized under conditions that are either optimal for the synthesis and accumulation of the SL RNA and the SL intron (Low ionic strength buffer = 20 mM KCl, 3mM $MgCl_2$) or strongly inhibitory for the synthesis of the SL RNA (High ionic strenghth buffer = 90 mM KCl, 8 mM $MgCl_2$). Figure 9 shows the results of this assay. RNAse protection of RNA synthesized in high ionic strength buffer (lane H) exclusively generated RNA fragments of 125 nt whose size was diagnostic

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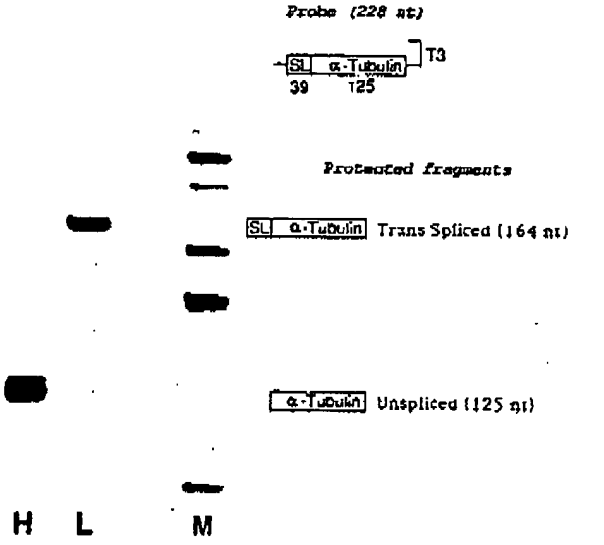

Figure 9. Detection of the trans-spliced 5′ end of mature α-tubulin mRNA. ^{32}P-RNA from 2.4×10^8 cells was synthesized using the ionic conditions described in the text. RNAse protection was performed as previously described (3) using 2 μg of biotinylated α-tubulin antisense RNA probe. The products of digestion were fractionated as described in Figure 1. Lane L: protected RNA fragments of RNA synthesized in low ionic strength buffer; lane H: protected RNA fragments of RNA synthesized in high ionic strength buffer; M, molecular weight marker. Diagrams of the α-tubulin antisense RNA probe and the expected products of RNAse protection are shown on the right. The probe is complementary to the mature 5′ end of the α-tubulin mRNA up to position 25 of the translated region (14). To clone the α-tubulin mRNA 5′ end, cDNA synthesis of trypanosome mRNA was primed with an oligonucleotide complementary to positions 1 to 25 of the α-tubulin translated sequence and the resulting cDNA was amplified by the polymerase chain reaction using the SL sequence as a second primer. The amplified DNA was inserted into the plasmid vector pT3T7 and its structure was verified by sequence analysis.

of unspliced α-tubulin RNA molecules. In contrast, the most abundant protected fragments obtained with RNA synthesized in low ionic strength buffer (lane L) had the size (164 nt) expected for accurately trans-spliced α-tubulin mRNA.

DISCUSSION

We have established conditions in permeable cultured procyclic trypanosomes which allow the cells to retain their ability to efficiently and accurately transcribe a variety of trypanosome genes and to trans-splice newly transcribed SL RNA and pre-mRNAs.

It was reported previously that lysolecithin permeabilization is compatible with a number of cellular functions including DNA synthesis (29), transcription initiation (30) and protein synthesis (29). Our results show that trans-splicing, an RNA processing reaction, is also carried out by detergent-treated cells. The detergent treatment is relatively mild and the overall cellular morphology and integrity of the plasma membrane are retained (ref. 29, and our unpublished observations). Nevertheless, after exposure to lysolecithin trypanosome cells become permeable not only to nucleotide triphosphates but also to macromolecules like DNAse I, which readily penetrates the nuclei and digests the nuclear DNA, and to DNA oligomers up to 23 nt long (33). These observations suggest that the intercalation of lysolecithin in the lipid bilayer has profound effects on the permeability characteristics of the cellular membranes. We are currently determining the size limits for penetration of proteins and nucleic acids into permeable trypanosomes.

In the permeble cell system described here, the efficiency of synthesis and accumulation of various cellular RNAs is severely affected by the concentrations of monovalent and, to a lesser extent, of divalent ions. Most surprising is the finding that potassium concentrations greater than 20 mM are strongly inhibitory for the synthesis of SL, U4, and 7SL RNAs (Figure 2A). Magnesium ions have less dramatic effects than potassium ions on the overall synthesis of SL, 7SL, U4 and tubulin RNAs (Figure 2B). However, above 3 mM [Mg^{++}] we note an accumulation of a 3′ end shortened form of the SL RNA (SL RNA 130). Preliminary pulse-chase experiments indicate that SL RNA 130 is derived from cleavage of intact SL RNA (unpublished observation). Thus, it seems plausible that magnesium concentrations between 5 mM and 12 mM primarily promote 3′ end cleavage of the SL RNA rather than inhibit its synthesis. Interestingly, SL RNA 130 accumulates *in vivo* when trypanosome cells are exposed to drugs that interfere with transcription and with RNA processing (20). Furthermore, SL 130 does not seem to be a substrate for trans-splicing (20). As Laird *et. al.* (20) pointed out cleavage of the SL RNA in the 3′ end domain might represent a way of marking the SL RNA for subsequent degradation by ribonucleases. High concentrations of magnesium might inhibit trans-splicing and this would channel the excess SL RNA towards degradation. Indeed, splicing of intervening sequences in HeLa cell nuclear extracts is inhibited at magnesium concentrations higher than 1.5 mM (31). As far as the effect of potassium is concerned, it seems likely that potassium ions primarily inhibit transcription of the SL RNA genes since no substantial amount of SL RNA 130 accumulates at suboptimal concentrations (Figure 1B).

Our conclusion that trans-splicing of newly synthesized RNAs is actively taking place in permeable trypanosomes is supported by the following observations. First, we detect the products expected from the cleavage of the SL RNA at the 5′ splice site, namely the SL exon and the SL intron. Second, branched RNA molecules consisting of the SL intron ligated to high molecular weight RNAs are found in newly-synthesized RNA from permeable cells. These forked molecules have been previously identified in steady-state RNA and represent putative intermediates in trans-splicing (4–6). Third, the majority of the newly transcribed α-tubulin RNA are trans-spliced, that is the SL sequence is joined to the α-tubulin mRNA body in the same configuration as in the mature mRNA. And finally, since the SL exon and the SL intron are detected using 1/20 of the amount of RNA required to detect the 5′ ends of α-tubulin mRNA, we argue that trans-splicing of α-tubulin RNA is not an exception and that a great number of trypanosome RNAs are trans-spliced in permeable cells.

Our results demonstrate that when the synthesis of the SL RNA and α-tubulin RNA are optimal, the correctly trans-spliced 5′ end of the α-tubulin mRNA is the predominant product present in newly-synthesized RNA. In contrast, when RNA is synthesized in buffers of high ionic strength and the synthesis of the SL RNA is inhibited, only unspliced α-tubulin RNA is produced. Since these latter conditions do not affect the transcription of α-tubulin RNA, this demonstrates that transcription and trans-splicing can be uncoupled. Furthermore, the finding that only unspliced α-tubulin RNA is synthesized in high salt buffers is in agreement with our previous findings concerning the synthesis of unspliced

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calmodulin RNAs in permeable trypanosomes (3). Indeed, the ionic conditions we used at that time were not optimal for SL RNA synthesis and, therefore, no trans-splicing of calmodulin RNAs was observed.

Although the free form of the SL intron has been identified in steady-state RNA by Sutton and Boothroyd (5), evidence from other authors (4) seems to point out that little or no free SL intron is detectable in trypanosome RNA. Our data clearly demonstrate that the newly-synthesized RNA derived from permeable trypanosomes contains large amounts of free SL intron which rapidly turns over (Figure 7). Under our experimental conditions the half life of the free SL intron is probably only a few minutes, but pulse-chase experiments are clearly required to obtain more precise measurements. Because the free SL intron is surprisingly abundant, we were concerned that its accumulation might be an artifact and not be related to trans-splicing activity. For instance, free SL intron might be derived from cleavage of the SL RNA by specific nucleases. Two different lines of evidence argue against this possibility. First, the time course of RNA synthesis shown in Figure 7 clearly shows that the amount of free SL intron in total RNA is not related to the amount of intact SL RNA. Second, when trans-splicing is inhibited by site-directed cleavage of trypanosome U snRNAs, no free SL intron accumulates (33). Therefore, we regard the appearance of the free SL intron as a sign of trans-splicing activity and, so far, we have observed 100% correlation between the two phenomena.

Pulse-chase experiments (6) and measurements of the decay rate of the SL RNA (20) demonstrate that the half life of the SL RNA is in the order of 4–6 minutes. In addition, the kinetic of accumulation of free SL intron suggests that trans-splicing of newly synthesized SL RNA can be detected in permeable trypanosomes as early as 5 minutes after transcription began. Therefore, the time required to trans-splice an individual pre-mRNA is probably very short and possibly less than a minute. How do trypanosomes achieve such a fast rate of trans-splicing? One possibility is that transcription and trans-splicing might occur simultaneously or, in other words, trans-splicing might take place co-transcriptionally. Interestingly, co-transcriptional cis-splicing seems to occur at a measurable frequency for the Drosophila chorion pre-mRNA (32). Tight coupling of transcription and trans-splicing would overcome the problem of delaying the process of 5′ end formation of trypanosome mRNAs, a process that in most eukaryotes is accomplished by transcription initiation. Because many trypanosome genes appear to be part of polycistronic or polygenic transcription units (1–3), one could speculate that trans-splicing and not transcription initiation is the rate limiting reaction for mRNA synthesis and that in trypanosomes the emphasis has shifted from transcriptional control to regulation of trans-splicing. The permeable cell system described here will allow us to study the physiology of the trans-splicing reaction in detail, to understand its relationship to transcription and to gain clues about its regulation.

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